Binding Sites for Neurotoxins and Cholinergic Ligands in Peripheral and Neuronal Nicotinic Receptors

Studies with Synthetic Receptor Sequences[a]

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The nicotinic acetylcholine receptors (AChRs) are formed by different combinations of homologous subunits: this is the structural basis for their pharmacological heterogeneity. The AChR has two binding sites for agonists and competitive antagonists that reside primarily on the two AChR α subunits. Inasmuch as the sites are at the interface between an α subunit and a neighboring one, non-α subunits also contribute to their formation and influence the pharmacological properties of the resulting AChR (for review see ref. 1).

The α subunit of *Torpedo* AChR was first identified as contributing structural elements of cholinergic sites recognized by snake α-neurotoxins, such as α-bungarotoxin (α-BTX), because *Torpedo* AChR expressed in *Xenopus* oocytes does not bind α-BTX unless the α subunit is expressed,[2] and the denatured isolated α subunits binds [^{125}I]α-BTX,[3–5] thus indicating that one or more continuous segment(s) of its sequence contributes to the α-BTX binding site.

In this paper we focus on the studies we carried out, using a synthetic peptide

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approach, on the structural elements contributed by the α subunits of different AChR isotypes to the binding sites for for α- and κ-neurotoxins from snake venoms, and for monoclonal antibodies (mAbs) able to compete with cholinergic ligands.

Using libraries of overlapping synthetic peptides corresponding to the deduced amino acid sequences of different AChR α subunits, we first identified sequence regions of different peripheral and neuronal α subunits able to bind cholinergic probes, such as α- or κ-neurotoxins or cholinergic competitive mAbs, in the absence of other surrounding structural elements (prototopes[6,7]), which are therefore likely to contribute to the formation of cholinergic binding sites. We then used single-residue substituted peptide analogues of these segments to identify structural requirements for binding of different cholinergic probes.[8–19]

These studies were carried out on different α subunit isotypes contributing to AChRs with different pharmacological properties. The results allow construction of possible models of the cholinergic sites, and shed light on the structural features underlying the pharmacological heterogeneity of peripheral and neuronal AChRs.

SUBUNIT COMPOSITION OF AChRs FROM PERIPHERAL AND NEURONAL TISSUES: DIFFERENT SUBTYPES OF α AND β SUBUNITS

AChR from peripheral tissues such as fish electric organ and vertebrate striated muscle comprise four subunits in a stoichiometry $\alpha_2\beta\gamma$(or ϵ)δ (reviewed in ref. 1). Different α and β subunit isoforms have been described in *Xenopus*[20] and/or mammalian muscle,[21,22] but their functional significance is not known.

Several AChR subunits have been identified and sequenced from neurons (reviewed in ref. 1). Neuronal AChR subunits can be classified, on the basis of the sequence homology among themselves and with the subunits of peripheral tissue AChRs, as α subunits, which contain within their N-terminal extracellular segment a vicinal pair of cysteine residues, the hallmark of all AChR α subunits, and β (or non-α) subunits. Conventionally, muscle AChR subunits are indicated with the postscript 1 (α_1, β_1), neuronal subunits with postscript numbers that indicate the order in which a particular subunit was identified and sequenced. Neuronal subunits from α_2 to α_9 and from β_2 to β_5 have been described thus far (reviewed in ref. 1).

Subunits corresponding to the muscle γ, ε, and δ subunits have not yet been described in neuronal systems. Expression studies demonstrated that, whereas muscle AChRs contain four different subunits, neuronal AChRs may contain only two subunits, α and β, or even one subunit only (α_7) (reviewed in ref. 1).

FUNCTIONAL HETEROGENEITY IN ACh SUBTYPES INDUCED BY DIFFERENT SUBUNIT COMBINATIONS

Functional diversity conferred by different combinations of subunits was first described for the AChRs of embryonic and adult mammalian muscle (reviewed in ref. 1). Coexpression of different neuronal α and β subunits in *Xenopus* oocytes also results in AChR having different conductance, open times and burst kinetics, and different pharmacology (reviewed in ref. 1).

The α subunit subtype is most important in determining the differential sensitiv-

ity of the resulting AChR complex to neurotoxins from invertebrates and snake venoms. Two classes of snake neurotoxins from the venoms of *Bungarus multicinctus, Bungarus flavus,* and different *Naja* species distinguish between AChR subtypes, that is, α-neurotoxins, such as α-BTX and α-najatoxin (α-NTX), and κ-neurotoxins, such as κ-bungarotoxin (κ-BTX) and κ-flavitoxin (κ-FTX) (reviewed in ref. 23) κ-BTX and α-BTX were initially regarded as specific antagonists of neuronal AChRs and muscle AChRs, respectively (reviewed in ref. 23). Later studies, however, revealed that this simple dichotomy does not hold true (reviewed in refs. 1 and 23).

α-BTX irreversibly blocks [$t_{1/2}$ > 200 h] AChRs formed by the subunit combinations $\alpha_1\beta_1\gamma\delta$ and $\alpha_1\beta_2\gamma\delta$ (reviewed in ref. 1). Several neuronal AChRs comprising different α/β subunit combinations—$\alpha_2\beta_2$, $\alpha_3\beta_2$, $\alpha_4\beta_2$, and $\alpha_3\beta_4$—are insensitive to α-BTX (reviewed in refs. 1 and 23). Neuronal AChRs formed by $\alpha_3\beta_2$ and $\alpha_4\beta_2$ subunit combinations are sensitive to κ-BTX.[24] The sensitivity of the $\alpha_3\beta_2$ AChR to κ-BTX is 10-fold greater than the $\alpha_4\beta_2$ complex.[25,26] Interestingly, the $\alpha_3\beta_4$ complex is insensitive to κ-BTX,[27] indicating that the β subunit affects the pharmacological properties of the resulting AChR. The $\alpha_2\beta_2$ neuronal AChR is insensitive to both α-BTX and κ-BTX.[28]

The chicken α_7 subunit can form homomeric AChRs sensitive to α-BTX (reviewed in ref. 1). However, the α_7 subunit may also contribute to formation of α-BTX insensitive AChR in the chick sympathetic ganglia, and therefore might also form AChR complexes, perhaps involving other α subunit subtypes, with different α-BTX binding properties than the complexes formed by α_7 subunit alone.[29]

Insect neuronal AChRs are sensitive to α-BTX (reviewed in ref. 1). Locust AChRs seem to be homomeric complexes of one type of subunit, although in *Drosophila* they may comprise both α and non-α (also known as β) subunits (reviewed in ref. 1).

The α subunit present in an AChR influences the sensitivity of the resulting complex to other neurotoxins. For example, neosugarotoxin, from the Japanese ivory shell, blocks AChRs formed by the β_2 subunit in combination with the α_2, α_3 or α_4 subunit, whereas the $\alpha_1\beta_1\gamma\delta$ AChR is relatively insensitive.[25,26] In contrast, α-conotoxins from the venom of marine snails block only the $\alpha_1\beta_1\gamma\delta$ AChR.[30] Lophotoxin, a cyclic diterpene from gorgonian corals, covalently labels Tyr_{190} of the *Torpedo* α subunit[31]—a residue conserved in all AChR α subunits but the neuronal α_5 subunit. As predicted from the presence of this tyrosine, AChR formed by combinations of $\alpha_1\beta_1\gamma\delta$, $\alpha_2\beta_2$, $\alpha_3\beta_3$, and $\alpha_4\beta_2$ subunits are sensitive to lophotoxin, although the $\alpha_2\beta_2$ AChR is less sensitive for reasons that remain unclear.[25] TABLE 1 summarizes the toxin sensitivity of AChRs resulting from combinations of different neuronal subunits.

USE OF THE SYNTHETIC SEQUENCE OF THE AChR α SUBUNIT FOR STRUCTURAL STUDIES OF CHOLINERGIC SITES: ADVANTAGES AND CAVEATS

We extensively used synthetic AChR sequences to investigate the structure of AChR domains which interact with the snake α- and κ-neurotoxins, and with mAbs against the cholinergic site.

Using peptides for those purposes required careful assessment of the legitimacy of those approaches, because most surface domains of proteins are formed by residues from discontinuous sequence regions, whereas synthetic peptides can be

successfully used to map tridimensional protein domains only if a substantial portion of the surface domain to be studied is formed by residues within the same short sequence segment. If folded properly, the corresponding synthetic sequence may then be representative of the structure of the domain, or of part of it.

That this can commonly occur in large proteins has been proven by a study that systematically investigated the ability of mAbs against different segments of the *Torpedo* AChR α-subunit sequence to cross-react with native AChR.[32] Monoclonal antibodies raised against three particular sequence segments of the α subunit fully cross-reacted with native AChR, indicating that those sequence regions are largely exposed on the AChR surface. By analogy, one would expect that the same will apply to most large proteins.

Protein ligands recognizing surface regions that include largely exposed sequence segments should be able to interact with the corresponding synthetic sequence. Therefore, synthetic peptides may be more confidently used to study the structural

TABLE 1. Contribution of Different Subunits to the Toxin Sensitivity of Neuronal AChRs

AChR	Neurotoxin Sensitivity
α1β1γδ α1β2γδ α7	α-BTX sensitive[24,71,72]
α2β2 α3β4	α-BTX insensitive, κ-BTX insensitive[24,27,28]
α3β2 α4β2	α-BTX low sensitivity, κ-BTX sensitive[24–26]
α2β2 α3β2 α4β2	Neosugarotoxin sensitive, α-conotoxin insensitive[25,26]
α1β1γδ	Neosugarotoxin insensitive, α-conotoxin sensitive[25,26]
α1β1γδ α2β2 α3β3 α4β2	Lophotoxin sensitive[25,26]

domain interacting with protein ligands than with small ligands, because high affinity protein/protein binding generally involves large surface interactions.

Experimental strategies employing a sequence region excised from the structural context of the cognate native protein have other important caveats. First, a sequence segment containing several residues involved in formation of a binding site may fold, in the absence of the structural constraints of the native protein, in a manner incompatible for ligand interaction, leading to false negative conclusions. On the other hand, one might obtain a positive result with a peptide corresponding to a sequence which in the native protein is inaccessible to the ligand because of obstruction by surrounding residues.

The latter possibility is exemplified by the findings that although coexpression in *Xenopus* oocytes of the α_3 subunit—which contains important constituent elements of cholinergic binding sites—together with the β_2 subunit results in an AChR

capable of irreversibly binding the neuronal AChR-specific antagonist κ-BTX (reviewed in refs. 1 and 23), expression of the α_3/β_4 subunit pair yields an AChR unable to bind κ-BTX,[27] or able to bind if only reversibly,[33] demonstrating that the β subunit contributes elements to the cholinergic site formation which interfere with the binding of κ-BTX to the α_3 domain. Similarly, the α_7 subunit contains a prototope for α-BTX binding,[14] and may form homomeric AChR complexes sensitive to the α-BTX (reviewed in ref. 1). However, the α_7 subunit may also be part of heteromeric AChRs that are insensitive to α-BTX.[34] Also, a prototope for α-BTX has been identified on the rat α_5 subunit,[9] which may not actually bind cholinergic ligands when part of a native neuronal AChR.[35]

Despite their potential pitfalls, studies with synthetic AChR sequences have proven to have a reliable predictive value (reviewed in ref. 36). Synthetic peptide studies of the main immunogenic region (MIR)—the set of largely overlapping epitopes on the extracellular surface of the AChR α subunit recognized by the majority of autoimmune antibodies (Abs) in the human disease myasthenia gravis,[37] correctly located the MIR to the sequence α67–76 on human muscle and *Torpedo* α subunits, as verified by comparisons of naturally occurring mutations in AChRs which do not bind anti-MIR Abs, and by the results of expression studies of mutant AChRs (reviewed in ref. 36). The sequence region that surrounds two vicinal cysteine residues, at positions approximately 192 and 193—the hallmark of AChR α subunits—has been identified as contributing to the formation of the α-BTX binding site by several studies employing synthetic and biosynthetic peptides (reviewed in ref. 1). This identification is supported by the results of studies using mutated AChRs, or cholinergic affinity ligands (reviewed in refs. 1 and 36). The results of studies using single-residue substituted peptides to identify residues within that sequence region important for AChR/α-BTX interactions,[11,18] agree well with those of *Xenopus* oocyte expression studies of mutated AChRs.[38]

Consistently, the affinity of the cholinergic probes for synthetic AChR sequences is several orders of magnitude lower than that for the native AChR. This is to be expected, because even when a short sequence region contributes several residues to a surface domain, other residues are contributed by discontinuous sequence regions (reviewed in ref. 39). That this is the case for the α- and κ-neurotoxin binding site is supported by the large toxin surface area involved in interaction with the AChR (reviewed in refs. 1 and 36) and the extremely high affinity for the AChRs of these toxins,[23] whose structural basis must be the intimate complementarity of large surface regions.

This reduced affinity of peptides for synthetic sequences excised from the context of the native protein may be beneficial when investigating individual residues involved in formation of a binding site by the use of single-residue substituted analogues: the relatively low affinity of the ligand/peptide interaction makes this a sensitive system to small changes in affinity. The same changes might be difficult to detect for substitutions of the same residues in the native AChR, given the very high binding affinity, and the stabilizing influence on the binding of the many more residues in the native AChR than in the peptide.

Solid phase assays, due to immobilization of the peptide sequence in a conformation noncompatible for ligand binding, might give falsely negative results. Therefore, it is advisable to use more than one type of solid phase assay and, whenever possible, a competition assay using peptides in solution. The latter assay, although more labor intensive than direct solid-phase binding assays, has the advantage that the peptide in solution may fold into any low energy conformation, which may include one corresponding to that in the native protein.

IDENTIFICATION OF SEQUENCE SEGMENTS CONTRIBUTING TO CHOLINERGIC SITES ON THE α SUBUNIT OF *TORPEDO* AND MAMMALIAN MUSCLE AChR

Several studies using synthetic or biosynthetic AChR sequences have indicated that the sequence region of the *Torpedo* α subunit flanking the vicinal Cys residues at position 192 and 193 forms a prototope for α-BTX (reviewed in ref. 1). We further defined structural elements of α-BTX binding site(s) on *Torpedo* AChR, by using a panel of overlapping synthetic peptides corresponding to the complete α subunit sequence as representative structural elements of the AChR.[8] We investigated whether, in addition to the sequence flanking the cysteinyl residues at positions 192 and 193, other sequence regions of the AChR α subunit could bind α-BTX, and/or several mAbs able to compete with α-BTX and with other cholinergic ligands for AChR binding. We also used overlapping peptides corresponding to the sequence segments of each *Torpedo* AChR subunit homologous to α166–203.

The mAbs used (WF6, WF5, and W2) were raised against native *Torpedo* AChR and specifically recognize the α subunit.[40,41] The binding of WF5 and W2 to *Torpedo* AChR is inhibited by all cholinergic ligands, that of WF6 by agonists, but not by low molecular weight antagonist.[40,41] The differential competition between the mAbs and cholinergic ligands, and the incomplete mutual inhibition by these mAbs for AChR binding, suggest that they bind to distinct overlapping parts of the area recognized by cholinergic ligands, and that within this area subsites may exist, recognized either by all small cholinergic ligands or by cholinergic antagonists alone. Binding subsites for different cholinergic ligands were suggested long ago, based on the results of pharmacological studies.[42]

α-BTX and WF6 bound to the synthetic sequence α181–200 and also, albeit to a lesser extent, to α55–74. The two other mAbs predominantly bound to α55–74, and to a lesser extent to α181–200. Peptides α181–200 and α55–74 both inhibited binding of [^{125}I]α-BTX to native *Torpedo* AChR. None of the peptides corresponding to sequence segments from other subunits bound α-BTX, WF6 or the other mAbs, or interfered with their binding.

Interestingly, results of studies from our and other laboratories[43,44] on the binding of α-NTX to synthetic sequences of *Torpedo* electric organ and mammalian muscle AChRs indicated that α-NTX, in spite of its high homology with α-BTX, with which it fully competes for binding to native AChR, does not recognize synthetic peptides corresponding to the sequence region flanking the cysteine residues 192–193. On the other hand, α-NTX binds effectively to the synthetic sequence α55–74 of *Torpedo* and muscle AChRs.[44] Therefore, as with the different mAbs against the cholinergic site described above, also highly homologous, mutually competitive α-neurotoxins may recognize different overlapping regions of the same binding area.

These results indicate that the cholinergic binding site is not a single narrow sequence region, but rather that two or more discontinuous sequence segments within the N-terminal extracellular region of the AChR α subunit, folded together in the native structure of the receptor, contribute to form a cholinergic binding region. Such a structural arrangement is similar to the "discontinuous epitopes" observed by X-ray diffraction studies of Ab-antigen complexes.[39] The multipoint attachments of α-BTX to the α subunit gives a structural basis for the high affinity of the α-neurotoxin/AChR interaction.

The structural characteristics of the *Torpedo* peptides α55–74 and α181–200 have been studied by circular dichroism (CD) and fluorescence spectroscopy (reviewed in refs. 1 and 11). Both peptides have a high content of β-sheet and β-turn. Differential CD-spectroscopy, in the presence and absence of α-BTX, indicates that peptides

α55–74 and α181–200 undergo structural changes upon α-BTX binding, with a net increase in the β-structure component (reviewed in refs. 1 and 11). These structural changes may reflect a mechanistic basis for the essentially irreversible inactivation of the AChR by α-BTX.

Similar studies carried out with panels of synthetic peptides corresponding to the complete α subunit sequence of mouse and human AChR demonstrated that only the sequence region flanking the vicinal cystines 192–193 forms a prototope for α-BTX.[13] Lack of detectable binding of α-BTX to peptide sequences of human and mouse AChRs homologous to the sequence *Torpedo* α55–74, which binds α-BTX, may be due to different reasons, including (1) improper folding of the peptide, different from that of the same sequence in the native AChR; (2) mutation in the mammalian muscle sequence region α55–74 of amino acid residues directly involved in α-BTX binding, and reduction of the affinity of this prototope for α-BTX to levels incompatible with detection of α-BTX binding in the assays used; and (3) lesser contribution to the structure of the α-BTX binding site by the sequence region α55–74 in mammalian muscle AChR than in *Torpedo* AChR.

IDENTIFICATION OF SEQUENCE SEGMENTS CONTRIBUTING TO CHOLINERGIC SITES ON THE α SUBUNIT OF κ-NEUROTOXIN SENSITIVE NEURONAL AChRs

Neuronal AChRs containing the α_3 subunit bind κ-neurotoxins, either irreversibly or reversibly (reviewed in refs. 1 and 33). Using overlapping peptides corresponding to the α_3 subunit sequence, we mapped a potential constituent segment of the binding sites to the sequence region $\alpha_3$51–70.[10,16] κ-BTX and κ-FTX bind to this sequence specifically; α-BTX does not bind to any α_3 peptides. The sequence $\alpha_3$51–70—which largely overlaps the homologous sequence regions of *Torpedo* AChR, α55–74, which forms a prototope for α-BTX (see above)—contains several negatively charged residues that may interact with the K and R residues present in the sequence loops of κ-BTX believed to interact with the AChR. It also contains several aromatic amino acids, which are a consistent structural feature of the α- and κ-neurotoxin binding prototopes (see below).

Two other largely overlapping peptide sequences that bind κ-BTX, $\alpha_3$180–199 and $\alpha_3$183–201, were identified using a competition assay with native neuronal AChR on PC-12 cells (most likely the $\alpha_3\beta_2$ subtype).[10] Both peptides contain the vicinal Cys pair, and are homologous to, although relatively divergent from, the muscle-type α-BTX binding sequence $\alpha_1$181–200 of different species. Therefore, in the α_3 neuronal AChRs, the sequence region surrounding the vicinal cysteines is also likely to contribute to the cholinergic binding site. An involvement of this region of the α_3 subunit in κ-BTX binding is supported by *Xenopus* oocyte expression studies using α_2/α_3 chimeras.[45]

Thus, like α-BTX, κ-neurotoxins appear to have multipoint attachments to the α_3 subunit, and the segments of the α_3 subunit contributing to these binding sites are homologous to those contributing to the α-BTX site in the *Torpedo* α subunits.

IDENTIFICATION OF SEQUENCE SEGMENTS CONTRIBUTING TO CHOLINERGIC SITES ON THE α SUBUNITS OF NEURONAL AChRs SENSITIVE TO α-BTX

We used similar experimental strategies to identify prototopes for α-BTX in two neuronal α subunits, the α_7 and α_8.[14] The cDNAs for these neuronal α subunits were

isolated using oligonucleotides corresponding to the aminoterminal sequence region of an α subunit from an AChR protein(s) isolated from chick brain using α-BTX affinity chromatography.[46] The α_7 subunit forms homo-oligomeric AChRs able to bind to α-BTX (reviewed in ref. 1); the α_8 subunit is highly homologous to the α_7 subunit.[47] We synthesized a panel of overlapping synthetic peptides and tested them for ability to bind [^{125}I]α-BTX. The peptides were 20 residues long, and corresponded to the complete chick brain α_7 subunit and to residues 166–215 of the chick brain α_8 subunit.[14]

We found that the synthetic sequences $\alpha_7$181–200 and $\alpha_8$181–200 consistently and specifically bound [^{125}I]α-BTX, although with different affinities—$\alpha_7$181–200 with an apparent affinity 10 times lower than that of $\alpha_8$181–200. The ability of these peptides to bind α-BTX was significantly decreased by reduction and alkylation of the Cys residues at positions 190/191, whereas oxidation had little effect on α-BTX binding activity.

Therefore, α_7 and α_8 are ligand binding subunits, able to bind α-BTX at sites homologous with the AChR α subunits from muscle and electric tissue, and the small sequence differences between these two highly homologous subunits may confer differential ligand binding properties to the AChR subtypes of which they are components.

We have used synthetic peptides to obtain clues about the neurotoxin sensitivity of neuronal AChRs containing an α subunits not successfully expressed in *Xenopus* oocytes as functional complexes, that is, the α_5 subunit.[9] Expression of α_5 mRNA correlates with the presence of neuronal α-BTX binding AChRs in several cell lines.[48] On the other hand, the α_5 subunits may contribute, together with the α_3 and β_4 subunit, to neuronal AChRs expressed in sympathetic ganglia that are sensitive to κ-BTX and insensitive to α-BTX.[29]

Overlapping peptides corresponding to the sequence region 171–205 of the α_5 subunit, of mouse muscle α_1 and rat neuronal α_2, α_3, and α_4 subunits, which all contain the vicinal Cys residues, were compared for ability to bind α-BTX.[9] In a solid phase assay testing the direct binding of [^{125}I]α-BTX to synthetic peptides, as well as in two different competition assays in which peptides were tested for their ability to sequester [^{125}I]α-BTX from binding to native AChR on postsynaptic membrane fragments of *Torpedo* electric organ or PC-12 cells, only peptides corresponding to the mouse muscle α_1 and rat neuronal α_5 subunits bound α-BTX.[9] These results are consistent with the known pharmacology of the α_1, α_2, α_3, and α_4 AChR subtypes (reviewed in ref. 1), and suggest that the α_5 subunit could bind α-BTX, and that the sequence $\alpha_5$180–199 may contribute to an α-BTX binding site. It is unclear in view of the results summarized above, whether the α-BTX binding prototope $\alpha_5$180–199 contributes in the cognate native neuronal AChRs to a cholinergic site able to bind α-BTX, or if in such native AChRs binding of α-BTX is impeded by surrounding structural elements.

IDENTIFICATION OF INDIVIDUAL RESIDUES INTERACTING WITH α- AND κ-NEUROTOXINS BY THE USE OF SYNTHETIC SEQUENCES: GENERAL CONSIDERATIONS

Following identification of AChR α subunit prototopes for binding of α- and κ-neurotoxins and of competitive mAbs, synthetic peptide approaches have also proven useful in identifying individual amino acid residues involved in the binding of those cholinergic probes.

We used two different approaches for these purposes. In the first, binding of

α-BTX to synthetic peptides corresponding to homologous prototopes of different α-BTX binding peripheral and neuronal AChR was studied to obtain clues about the effect of naturally occurring amino acid substitutions on α-BTX binding. As described below, those studies demonstrated a lack of sequence motifs identifying α-BTX binding proteins. Therefore a second approach was used in attempts to determine the structural requirements for α-BTX and κ-neurotoxin binding, that is, investigation of the effect of individual nonconservative or conservative substitutions on the binding of α-BTX or competing mAbs, or κ-neurotoxins, to the sequence regions of peripheral or neuronal AChRs found to bind these cholinergic probes. The results of these studies will be compared and, if necessary, contrasted with those of investigations employing mutation analysis of AChRs expressed in oocytes.

COMPARISON OF HOMOLOGOUS SEQUENCES α181–200 FROM MUSCLE AChR OF DIFFERENT SPECIES

Muscle AChR α subunits are highly conserved between different species. Because the sequence α181–200 in *Torpedo* electric organ, mouse and human muscle AChRs forms a prototope for α-BTX, we systematically investigated the ability to bind α-BTX and the affinity of such binding of synthetic peptides that correspond to the homologous sequence regions from the muscle of other vertebrate species, whose AChR α subunit sequence is known.[13] Included in the species studied were the sequence regions α181–200 of *Naja* and mongoose muscle AChRs. These animals are notable exceptions to the rule that α-BTX blocks muscle AChR function: several snake species, including *Naja,* are resistant to the blocking action of α-BTX,[49] and the mongoose, a mammal well known for its ability to kill and eat poisonous snakes (e.g., see ref. 50), is also resistant to snake neurotoxins.[51]

All the synthetic sequences α181–200 tested contained an α-BTX binding prototope, with the notable exceptions of the *Naja* and the mongoose sequences.[13] As illustrated in FIGURE 1, the sequence region α181–200 is very conserved in *Torpedo* and vertebrate muscle AChRs. However, this does not apply to the *Naja* and mongoose sequences. Six amino acid residues of this sequence region in the snake α subunit differ from the other α subunits, and may be important for α-BTX binding. Nonconservative substitutions in the snake α181–200 sequence include replacements of K_{185}, W_{187}, Y_{189}, and P_{194} by W, S, N, and L, respectively. Single residue mutations of the *Torpedo* α sequence to each of the six substitutions of the *Naja* α sequence demonstrated that conversion of Y_{189} to N or P_{194} to L in the *Torpedo* sequence suffices to eliminate α-BTX binding.[52] In the mongoose sequence, W_{187}, Y_{189}, and P_{194} are nonconservatively substituted to N, T, and L respectively.[49] In addition, P_{197}, conserved in all known peripheral AChR α subunits, is nonconservatively substituted to H, and at position 195 the negatively charged residue present on most α-BTX binding prototopes and proposed to be involved in interaction with α-BTX,[53] is conservatively substituted by T.[49]

Comparison of the vertebrate muscle α sequences (FIG. 1) shows that the sequence VVY at positions 188–190 is common to the peptides that bind α-BTX with high affinity (*Torpedo,* frog, chick), and that substitution of the amino acid at position 189 to F (as in the calf and mouse sequences), N (as in the *Naja* sequence) or T (as in the human and mongoose sequences) correlates with a reduced affinity of this prototope for α-BTX.[15] The convergence of these results strongly indicates Y_{189} as a critical residue in the interaction of α subunits from different muscle AChRs with α-BTX.

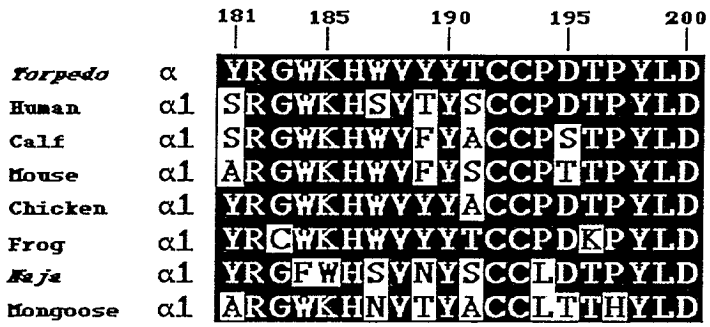


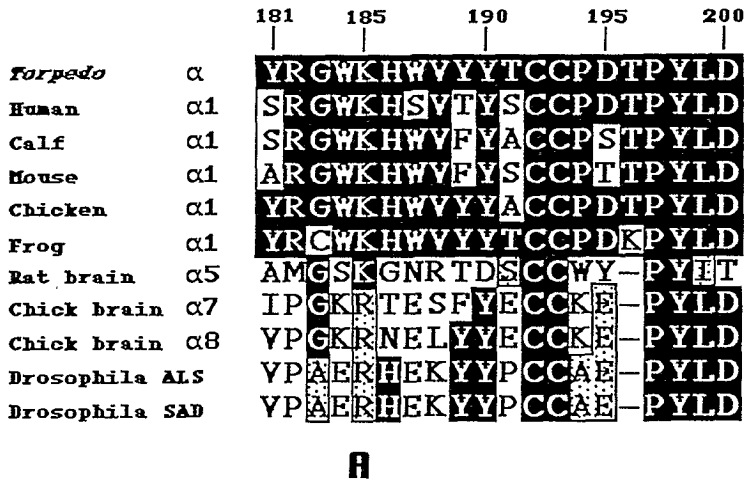
FIGURE 1. Alignment of the sequence region 181–200 from the α subunit of different vertebrate muscle AChRs. This sequence region is highly conserved in most muscle α subunits. Identical residues (as compared to the *Torpedo* sequence, which is aligned at the top of the sequences) are enclosed in black boxes. The amino acid residues which are substituted, as compared to the *Torpedo* α subunit, are in white boxes. Several nonconservative substitutions within this region are present in the α subunits of cobra and mongoose muscle AChRs. This may be related to the resistance of muscle from these species to α-neurotoxin block, and the inability of synthetic peptides corresponding to the cobra and mongoose sequence region α181–200 to bind α-BTX. See text for further details. (From Conti-Tronconi *et al.*[1] Reproduced with permission of the *CRC Critical Reviews in Biochemistry and Molecular Biology.*)

COMPARISON OF HOMOLOGOUS SEQUENCES α181–200 FROM NEURONAL AChRs OF DIFFERENT SPECIES AND SUBTYPES

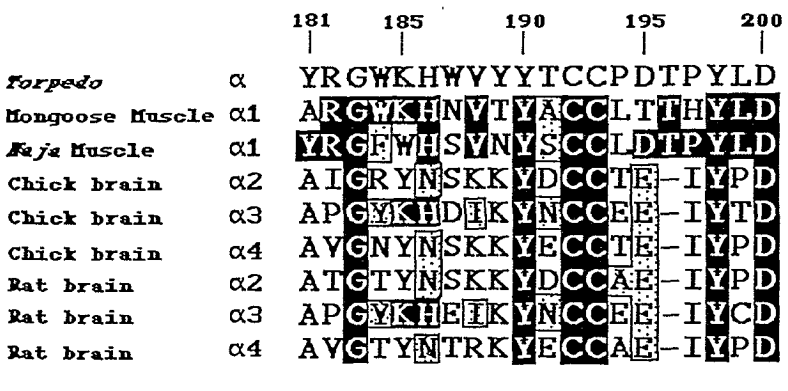
The sequence region surrounding the vicinal cysteines 192/193 of the neuronal α-BTX binding α subunits is highly diverged with respect to the *Torpedo* and muscle α1 subunits (FIG. 2). The low predictive value of sequence homology to infer neurotoxin sensitivity can be best appreciated by comparison of the sequence regions of AChR α subunits that bind α-BTX (*Torpedo* α, vertebrate muscle α_1, rat neuronal α_5, chick brain α_7 and α_8, and *Drosophila* ALS and SAD subunits), with the homologous sequence regions of AChRs that do not bind α-BTX (the *Naja* and mongoose muscle α_1, and the neuronal α_2, α_3, and α_4 subunits). Seven amino acid residues are characteristic of all α subunits regardless of their α-BTX binding ability: that is, G_{183} (or the conservative substitution A), Y_{190}, C_{192}, C_{193}, D_{195} (or the conservative substitution E), Y_{198}, and D_{200}. All of the α subunits in FIGURE 2 that bind α-BTX have Y_{189} (or the conservative substitution F) and P_{197}, whereas K_{189} and I_{197} are characteristic of α subunits that do not bind α-BTX. This general rule, however, does not hold true for the α_5 subunit, whose sequence is highly divergent from other AChR α subunits.

The inability to correlate critical structural features required for α-BTX binding with a particular amino acid sequence indicates in a broader sense a serious limitation to the use of sequence homology to define families of functionally and structurally related proteins. It is obvious from comparison of the α-BTX binding sequences that different primary sequences must fold into three-dimensional structures with comparable hydrophobic, hydrogen-bonding, and charge interactions. Compensatory, multiple nonconservative substitutions that occurred during the evolution of α-BTX binding proteins have obscured a "universal" α-BTX binding motif. This fact is also illustrated by the lack of sequence homology between any of

the nicotinic AChR α subunits and the ACh binding sites of the muscarinic ACh receptor and acetylcholinesterase.[54,55] The failure to find a common α-BTX binding motif is similar to the search for targeting sequence signals involved in sorting proteins into different cellular compartments. In those cases, instead of primary



A



B

FIGURE 2. Alignment of the sequence region 181–200 from the α subunit of different muscle and neuronal AChRs. The residues are numbered with reference to the *Torpedo* α subunit sequence. Identical residues (as compared to the *Torpedo* sequence, which, for sake of comparison, is also aligned at the top of the sequences that cannot bind α-BTX) are enclosed in black boxes, conservative substitutions on a dotted background. (**A**) AChR α subunit sequences that bind α-BGT; (**B**) AChR α subunit sequences that do not bind α-BGT. See text for further details. (From Conti-Tronconi *et al.*[1] Reproduced with permission of the *CRC Critical Reviews in Biochemistry and Molecular Biology.*)

sequence conservation, compositional motifs are found, in which certain amino acids or residues with similar physical characteristics are common between proteins destined to the same cellular organelle or membrane compartment (see ref. 56).

INDIVIDUAL RESIDUES OF THE *TORPEDO* α SUBUNIT SEQUENCE INTERACTING WITH α-NEUROTOXINS: STUDIES WITH SINGLE RESIDUE SUBSTITUTED PEPTIDE ANALOGUES

We attempted to elucidate the structural requirement for ligand binding to the cholinergic subsites formed by the sequences α181–200 and α55–74 of *Torpedo* AChR by investigating the effect on the binding of α-BTX of nonconservative and conservative substitutions of individual residues within these sequence regions. For the sequence region α181–200, we also investigated the binding of mAb WF6 and for α55–74, of α-NTX.

We first used a panel of substituted peptide analogues of the *Torpedo* sequence α181–200, carrying single amino acid substitutions of glycine or alanine for each native residue.[11] Circular dichroism spectral analysis indicated that the substituted analogues had comparable structures in solution, and they could therefore be used to analyze the influence of single amino acid substitutions on ligand binding. Several peptide analogues clearly differed from the unsubstituted parental sequence in their ability to bind α-BTX or mAb WF6, or both.

Distinct clusters of amino acid residues, discontinuously positioned along the sequence α181–200, seem to serve as attachment points for the two ligands studied. The residues necessary for binding of α-BTX are different from those crucial for binding of WF6. In particular, residues at positions 188–190 (VYY) and 192–194 (CCP) were necessary for binding of α-BTX, whereas residues W_{187}, T_{191}, and Y_{198} and the three residues at positions 193–195 (CPD) were necessary for binding of WF6. Several other residues flanking the two clusters VYY and CCP also seemed to be involved in α-BTX binding (W_{184}, K_{185}, W_{187}, D_{195}, T_{196}, P_{197}, and Y_{198}).

Comparison of the CD spectra of the toxin/peptide complexes, and those obtained for the same peptides and α-BTX in solution, indicates that structural changes of the ligand(s) occur upon binding, with a net increase of the β-structure component. The increase in the order of the structure may reflect a structural rearrangement of the peptide upon binding to the high affinity α-BTX "matrix."

These results further demonstrate that within this relatively large structure, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of the attachment points exist. This may be the molecular basis of the wide spectra of binding affinities, kinetic parameters, and pharmacological properties observed for the different cholinergic ligands.

The results described above were verified by other studies. A study using single amino acid substitutions (to G or A) of the sequence segment *Torpedo* α188–197, indicated that Y_{189}, Y_{190}, and D_{195} are important for α-BTX binding.[53] Another study, which tested the effect of multiple amino acid substitutions of the *Torpedo* sequence α166–211 expressed as a bacterial fusion protein, confirmed that Y_{189} and P_{194} are critical for α-BTX binding.[52]

Other studies investigated the binding of α-BTX and ACh to mouse muscle α subunit mutants expressed as AChR complexes in *Xenopus* oocytes.[57] Although the affinity for ACh was markedly reduced when Y_{190} was substituted to F, none of the substitution administered affected the α-BTX binding detectably. The different results of this study with respect to the ability to bind α-BTX and of studies using

synthetic "mutated" sequences of *Torpedo* and mouse α subunits could be due to (1) species differences, or (2) differences in the amino acid substitutions made, or—most likely—(3) the fact that, because binding of α-BTX to native AChR occurs via large interacting surfaces, mutation of one of the several residues involved in such interaction may not suffice to change the binding affinity detectably.

To further define the structural requirements for α-BTX binding to the prototope α181–200 of *Torpedo* AChR, we investigated the effect on α-BTX binding to this synthetic sequence of conservative substitutions of residues that previous studies had indicated as important for binding of α-BTX.[18] Amino acid substitutions for this mutational analysis were chosen in order to determine (1) physico-chemical attributes of the amino acid side chains that mediate α-BTX binding (i.e., steric,

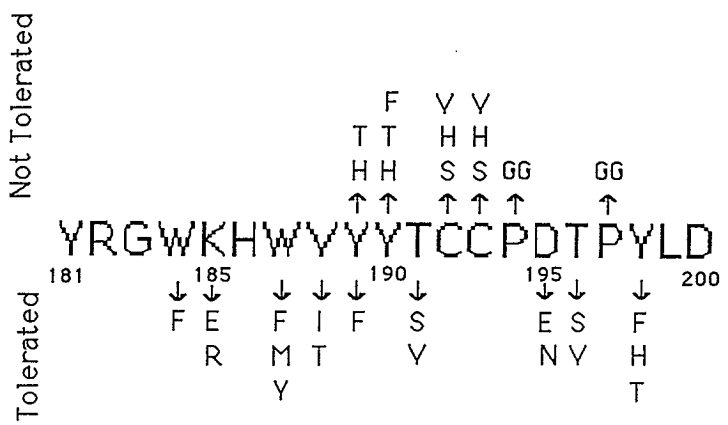


FIGURE 3. Summary of the effect of conservative amino acid substitutions on the binding of [^{125}I]α-BTX to *Torpedo* AChR α181–200. Consistent results obtained for both the solution and the solid phase assays are summarized. The native sequence of *Torpedo* α181–200 is indicated at the center of the figure, and the residue position numbers indicated under amino acids are relative to the *Torpedo* α subunit. The amino acid substitutions that are tolerated and result in equal or slightly lower α-BTX binding activity are indicated by arrows from below the positions of the native sequence, which are replaced. Similarly, those substitutions that are not tolerated and abolish α-BTX binding activity of the peptides are indicated by arrows above the native sequence. (From McLane *et al.*[18] Reproduced with permission of *Biochemistry.*)

hydrophobic, hydrogen-bonding, and/or electrostatic interactions) and (2) how the secondary structural constraints imposed by the presence of prolyl residues or a disulfide bond confer α-BTX binding activity to this prototope.

The conclusions of those studies,[18] based on consensus of solid and solution phase assays, are summarized in FIGURE 3. Amino acid substitutions that had a profound effect on α-BTX binding are indicated above the native *Torpedo* α181–200 sequence (Not Tolerated), and those that slightly or moderately affected α-BTX binding are indicated below the native sequence (Tolerated). Conservative substitutions of Y_{189}, Y_{190}, C_{192}, and C_{193} abolished or strongly affected α-BTX binding, whereas V_{188} could be conservatively replaced by I or T with minor effects on α-BTX binding. We previously suggested that these residues form important contacts with

α-BTX, based on the results obtained when these residues were substituted with G.[11] Structural changes of the peptide α181–200 induced by substitution of P_{194} or P_{197} with two adjacent G residues, or insertion of a G between C_{192} and C_{193}, were also incompatible with α-BTX binding. Conservative substitutions of other aliphatic and aromatic residues, and of residues K_{185} and D_{195}, had minor effects on α-BTX binding.

Therefore, binding of α-BTX to the prototope α181–200 involves important interactions with Y_{189}, Y_{190}, C_{192}, and C_{193} that are highly specific to the amino acid residues at these positions. Residues Y_{190}, C_{192}, and C_{193} are highly conserved, because the two vicinal C residues are the hallmark of the α subunits, and Y_{190} is found in all but the α_5 α subunits (reviewed in ref. 1). However, these three residues *per se* are not correlated with α-BTX binding activity, because they are conserved in α subunits that do not bind α-BTX (reviewed in ref. 1). The presence of Y_{189}, on the other hand, correlates with high-affinity α-BTX binding (reviewed in refs. 1 and 23). P_{184} and P_{187} play important structural roles in maintaining the correct conformation of the peptide to display the α-BTX binding motif.

α-BTX is believed to contact the AChR through a region of extensive β-sheet that forms a carapace, excluding water from the AChR/α-BTX interface.[58–60] It can be expected that the α-BTX binding site on the AChR itself will also be formed by a β-sheet. Both protopes α181–200 and α55–74 spontaneously fold in solution to form a β-sheet.[1,11] The results of our study using conservatively substituted analogues of the prototope α180–200 further support the possibility that a β-sheet folding of this sequence is necessary for α-BTX binding, because substitution of either C_{192} or C_{193} by residues with side chains of similar size (S and V) is not tolerated, nor is substitution by H, which shares polar and hydrophobic properties. C_{192} and C_{193} form a highly reactive vicinal disulfide in the native AChR (reviewed in ref. 1) which, however, is not required for α-BTX binding, either to native *Torpedo* AChR or to the synthetic sequence α181–200 (reviewed in ref. 1). Therefore, the important attributes of cysteinyl residues at these positions do not involve a propensity to form a vicinal disulfide, but rather are a function of the size and hydrophobic nature of the cysteinyl side chains.

Structurally, the importance of the C/C pair for α-BTX binding is also demonstrated by the inability to bind α-BTX of a peptide analogue of α180–200, where a glycine was inserted between C_{192} and C_{193}. The introduction of any conformational flexibility within this region is deleterious for α-BTX binding, as shown by the inability to bind α-BTX of peptide analogues of α180–200, where two glycines were substituted for either P_{184} or P_{187}.

Both vicinal disulfides and proline imides have a propensity to form nonplanar, *cis* peptide bonds.[61,62] Two adjacent *cis* peptide bonds could act together to cause a turn in the peptide backbone, providing an important element of secondary structure for α-BTX activity of this prototope. Molecular modeling of this region indicates that the vicinal disulfide, P_{194} and P_{197}, are likely to induce a β-turn.[63,64] This would allow the β-strand formed by this peptide to fold-back on itself and stabilize this conformation by β-sheet formation.[62] Such structure would complement the β-sheet folding of the binding area of the α-BTX molecule.

The guanidium group of R_{37} on α-BTX, common to all α-neurotoxins, may be analogous to the quaternary ammonium group of ACh and other nicotinic agonists and antagonists.[65] A complementary negative subsite on the α subunit of the AChR was proposed to involve D_{195},[66] but affinity labeling studies have indicated that this residue is not involved in cation stabilization of acetylcholine.[67] Our studies also indicated that D_{195} can be substituted by N with little effect on α-BTX binding.

An alternative model for a complementary binding subsite on the AChR α subunit for the quaternary ammonium of ACh is suggested by two other systems that indicate that cation stabilization can be provided by π-electrons from aromatic rings: (1) a synthetic ACh "receptor" composed entirely of aromatic rings[68] and (2) the structure of acetylcholinesterase, whose active site is at the bottom of a gorge lined with aromatic amino acids.[69] Thus, the AChR subsite required for cation stabilization might be provided by the π-electrons present in the numerous aromatic residues of both the prototopes α181–200 and α55–74 (see below). Many of those aromatic residues are crucial or important for α-BTX binding. Also, in neuronal AChR α subunits, the prototopes for α- and κ-neurotoxins are rich in aromatic residues, whose substitution is frequently poorly or not tolerated.

We used a similar approach to investigate the residues within the sequence *Torpedo* α55–74 involved in the binding of α-BTX and α-NTX.[17] This sequence region overlaps the sequence segment α67–76, which has been shown to contribute important structural elements of the MIR (reviewed in ref. 1). However, α-BTX and anti-MIR mAbs bind to the AChR in a nonmutually exclusive way, suggesting that the two sides do not overlap. We used a panel of single residue substituted analogues of the sequence Tα55–74 to identify the residues involved in α-neurotoxin binding and those involved in the binding of anti-MIR mAbs. Binding of α-BTX and α-NTX was similarly affected by substitutions within this sequence. The overlap between the residues important for α-neurotoxin binding and those involved in anti-MIR Ab binding was minimal. Substitution of several positively charged or aromatic residues strongly inhibited α-BTX binding; of those, only one (W_{60}) significantly reduced the binding of two anti-MIR mAbs. Only substitution of residue N_{68} strongly reduced the binding of both α-BTX and anti-MIR mAbs. These results are consistent with a model in which the MIR and the α-neurotoxin binding site, although within the same large surface area of the native AChR and very close to each other, have minimal overlap, and in which the α-neurotoxin binding site is rich in aromatic residues (see above).

RESIDUES OF THE NEURONAL α_5 SUBUNIT SEQUENCE INTERACTING WITH α-BTX: STUDIES WITH SINGLE RESIDUE SUBSTITUTED PEPTIDE ANALOGUES

The α-BTX binding sequence region 180–200 of the rat α_5 subunit is relatively divergent as compared with the homologous sequence regions of *Torpedo* and muscle AChRs. Amino acid residues critical for α-BTX binding were identified by testing the effects of single amino acid substitutions to G or A for each residue of the rat $\alpha_5$180–199 sequence on binding of α-BTX to the substituted peptide analogues.[15] Substitutions of four residues (K_{184}, R_{187}, C_{191}, and P_{195}) abolished α-BTX binding; other substitutions (G_{185}, N_{186}, D_{189}, W_{193}, Y_{194}, and Y_{196}) lowered its affinity. On the other hand, substitutions of C_{192} (homologous to C_{193} of *Torpedo* AChR α subunit) did not affect the ability of the α_5 prototope to bind α-BTX.

The lack of effect of substitution of one of the vicinal cysteines underlines that, as discussed above, a disulphide bridge between them is not crucial for α-BTX binding. Presumably, in the α_5 prototope the several nonconservative changes, as compared to the homologous *Torpedo* and muscle prototopes, stabilize its β-sheet conformation, even in the absence of the stabilizing action of a planar *cis*-disulphide bridge.

The importance of several aromatic amino acids for α-BTX binding to the α_5 peptide is analogous to the findings reported above for the *Torpedo* α180–200 sequence. Thus, despite the apparent divergence of the α_5 sequence from other α-BTX binding α subunits, some structural features, such as an abundance of aromatic residues and amino acids able to contribute electrostatic and/or hydrogen bond interactions, have been conserved.

RESIDUES OF THE NEURONAL α_3 SUBUNIT SEQUENCE INTERACTING WITH κ-NEUROTOXINS: STUDIES WITH SINGLE RESIDUE SUBSTITUTED PEPTIDE ANALOGUES

Synthetic peptide analogues of the sequence $\alpha_3$50–71, in which each amino acid was sequentially replaced by a glycine, were used to identify the amino acid side chains involved in the interaction of this prototope with κ-neurotoxins by testing their ability to bind [^{125}I]κ-BTX and [^{125}I]κ-FTX.[12,16] No single substitution obliterated κ-BTX binding, but several substitutions lowered the affinity of this peptide for κ-BTX—two negatively charged residues (E_{51} and D_{62}) and several aliphatic and aromatic residues (L_{54}, L_{56}, and Y_{63}).[12]

[^{125}I]κ-FTX binding was more sensitive to amino acid substitutions than that of [^{125}I]κ-BTX.[16] Similar to κ-BTX, aliphatic and aromatic amino acid residues were important for κ-FTX binding (L_{54}, L_{56}, and Y_{63}, also involved in κ-BTX binding, and additional W residues at positions 55, 60 and 67). In contrast to κ-BTX, however, positively rather than negatively charged amino acids appeared to mediate electrostatic interactions with κ-FTX—K residues at positions 57, 64, 66, and 68.

These differences in amino acid specificity can be correlated with sequence differences of κ-BTX and κ-FTX, and provide clues as to the reason for these different charge requirements and the residue interactions at the κ-neurotoxin subunit interface.

κ-FTX and κ-BTX are both highly basic (pI 8.8 and 9.1, respectively) and share 82% sequence identity: the different residues (12 out of 66) are clustered in two sequence regions believed to interact with the AChR, region I (positions 23–33) and region II (positions 43–54).[70] Region I of the homologous α-neurotoxins interacts with the peripheral AChR α subunit.[58–61] Within region I of the κ-neurotoxins, the amino acid substitutions are primarily conservative and do not result in differences in charge distribution. This region might determine the overall binding specificity of κ-FTX and κ-BTX for the sequence segment $\alpha_3$51–70. The amino acid substitutions of region II result in a different overall charge and differences in the spatial arrangement of charged groups. E48 of κ-FTX, which is Q in κ-BTX, may determine the relative importance for positively charged groups of the $\alpha_3$51–70 sequence for binding. The different spatial arrangements of an arginine residue (positions 50 and 52 of κ-BTX and κ-FTX, respectively) may account for the unique sensitivity of κ-BTX binding to substitution of negatively charged residues in the peptide $\alpha_3$51–70.

A common requirement for the binding of both κ-neurotoxins is the presence of several aliphatic and aromatic residues. These are features similar to those identified as structural requirements for the binding of α-neurotoxins to their AChR prototopes, supporting the notion that similar mechanisms may apply to the interaction of the different AChR isotypes with α- or κ-neurotoxins, and, therefore, that the cholinergic binding site may have similar structure in all AChRs.

SUMMARY OF THE RESULTS OF STUDIES ON THE STRUCTURE OF CHOLINERGIC BINDING SITES BY USE OF SYNTHETIC OR BIOSYNTHETIC PEPTIDES

The studies summarized in the previous sections allow the following conclusions:

1. At least two sequence segments of the α subunits, which always include the segment containing the vicinal cysteines, contribute to form the cholinergic binding sites recognized by α- and κ-neurotoxins. These sites therefore are complex surface areas, formed by clusters of amino acid residues from different sequence regions.
2. The sequence segments contributing to the cholinergic site are in similar positions along the α subunit sequences, suggesting that the extracellular domain of all α subunits folds in a similar manner.
3. The sequence region α180–200 is very conserved, and well defined clusters of residues surrounding and including the residues Cys_{192} and Cys_{193} are involved in interaction with α-BTX. The homologous sequence region is not well conserved in neuronal AChRs that bind α-BTX, and the residues identified as crucial for interaction with α-BTX are at positions different from those of peripheral AChRs. Therefore, there is no universal sequence motif with predictive value for α-BTX binding, and multiple, nonconservative substitutions in these sequence regions during evolution of the AChR proteins have both obscured the original ancestral sequence and reestablished, as a result of new mutual interactions, a structure compatible with α-BTX binding.
4. Although Cys_{192}/Cys_{193} are involved in forming the toxin/α-subunit interface, a vicinal disulfide bond is not required for α-BTX binding.
5. Within the relatively large area of the cholinergic site, cholinergic ligands bind with multiple points of attachment, and ligand-specific patterns of attachment points exist. This may be the molecular basis of the broad spectra of binding affinities, kinetic parameters, and pharmacological properties observed for the different cholinergic ligands.
6. The sequence regions α181–200 and α50–75 are unusually rich in aromatic residues, whose substitution frequently affects α- and κ-neurotoxin binding. These findings suggest that the anionic cholinergic binding site of the AChR is formed not by a single negatively charged residue, but rather by interaction of the π-electrons of aromatic rings, as demonstrated for the cholinergic site of acetylcholinesterase.

CONCLUSIONS

The structural requirements for α- and κ-neurotoxin binding to peripheral AChRs, as predicted from the x-ray structure of α-neurotoxins, and the deduced structure of the homologous κ-neurotoxin, include that the toxin/receptor interface is a large area composed of residues that are hydrophobic or aromatic. These structural predictions have been born out by studies employing synthetic peptides. Qualitatively similar results have been obtained by studying the binding of α- and κ-neurotoxin prototopes, supporting the notion that the cholinergic binding site may have similar structure in all AChRs.

We have summarized previously the several caveats of experimental approaches which employ synthetic sequences excised from the structural context of the cognate

protein, as representative structural elements of the native protein. Great caution must be exercised in extrapolating the results of low-affinity binding to synthetic sequences to the very high-affinity binding sites of the native, heterooligomeric complexes of the intact AChR. The relative contribution to binding of α- or κ-neurotoxins of an individual sequence segment observed in our experiments using peptides may not be representative of the accessibility or conformation of that sequence in the corresponding native AChR, or of its actual importance for high-affinity toxin binding to native AChRs. On the other hand, identification of potential sequence regions that might contribute to the cholinergic binding site on different AChR subtypes will make it possible to use targeted mutagenesis and expression of functional AChRs, to test the actual importance of these sequence regions in formation of the high-affinity sites in the intact receptor. This sort of information could ultimately be used for the rational design by protein engineering of peptide toxins specific for a given AChR subtype, which are not naturally occurring.

[**NOTE:** For recent reviews published by our group on these matters, see references 1 and 36; also, McLane, K. E., S. J. M. Dunn, A. A. Manfredi, B. M. Conti-Tronconi & M. A. Raftery, 1994, "The Nicotinic Acetylcholine Receptor as a Model of a Superfamily of Ligand-Gated Ion Channel Proteins," in Handbook for Protein and Peptide Design, P. R. Carey, Ed., Academic Press, Orlando, FL.

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